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Transposon Mutagenesis in Mycobacterium avium Subspecies Paratuberculosis

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Chapter 11

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Abstract

While transposon mutagenesis has been developed for *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), relatively few laboratories have adopted this important genetic tool to examine gene function and essentiality. Here we describe the construction of a *Map* transposon library using the *Himar1* mariner transposon, but concepts can also be applied to the Tn*5367* transposon, which has also been used by our group. Delivery of the transposon is by a temperature-sensitive phagemid, ϕMycoMarT7, and plating transductants requires patience and specialized media due to length of incubation required to observe colonies. Several transposon mutants obtained from these libraries have been tested in vaccine and pathogenesis studies. By providing the following detailed protocol herein, we expect to demystify the procedure and encourage additional investigators to incorporate transposon mutagenesis in their studies on Johne's disease.

Key words Transposon mutagenesis, Mycobacterium, Johne's disease

1 Introduction

Mycobacterium avium subspecies *paratuberculosis* (*Map*) causes Johne's disease in cattle, sheep, goats, and other ruminants. It is among the slowest growing mycobacteria with a generation time of approximately 24 h and some strains, especially those isolated from sheep, are difficult to culture. These factors have combined to delay advancements in genetic studies of *Map* relative to other mycobacteria including *M. smegmatis* and *M. tuberculosis*, especially as it relates to vaccine strain construction and essential gene analysis.

The first efforts to genetically modify *Map* came with the discovery that mycobacteriophage TM4 could form plaques on *Map* lawns [1]. Soon after, a TM4 derivative, phAE94, was engineered to deliver the Tn*5367* transposon randomly into the *Map* genome [2]. Tn*5367* is derived from IS*1096* from *M. smegmatis* [3]. With this technology in hand, a library of over 5000 transposon mutants was initially constructed [2], and later, 13,500 Tn*5367*

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mutants of *Map* were obtained [4]. Directed knockouts using insertion sequences have now also been achieved in *Map*. Examples of genes that have been targeted using this method include *sigH* [5, 6], *lipN* [5], *leuD* [7], *relA*, *lsr2*, and *pknG* [8], with *relA* [9] and *leuD* [10] mutants showing protection from *Map* challenge in goats.

More recently, our group compared the total genome insertions sites for the mycobacterial transposon Tn*5367* with the mariner transposon MycoMarT7 carrying the *Himar1* transposase [11]. The dinucleotide insertion recognition site (5′-TA-3′) for MycoMarT7 was significantly more prevalent than the degenerate tetramer (5′-NNPy[A/T]A[A/T]NN-3′) Tn*5367* recognition sites such that only 37 genes do not contain a MycoMarT7 site, compared to 710 genes missing Tn*5367* sites. Therefore, transposons containing the IS*1096* element have recognition site distribution biases that could affect the interpretation of gene essentiality on a whole genome basis. Nonetheless, this transposon has been useful for single gene studies and mutant library selection under defined conditions [4, 12–15].

Tn*5367* carries a kanamycin-resistance marker and can be delivered to *Map* by a thermosensitive phagemid phAE94 derived from mycobacteriophage TM4 [1, 16]. Whereas, MycoMarT7 is a *Himar1*-derived transposon that also carries a kanamycin-resistance marker engineered into the same TM4 phagemid, designated ϕMycoMarT7 [17, 18]. Here we describe the detailed protocols for obtaining a *Himar1* transposon library in *Map*.

2 Materials

For transposon mutagenesis, the only two strains of *Map* used thus far are the type strain (ATCC19698) and the bovine clinical isolate K-10. Other *Map* stains are expected to be amendable to the genetic manipulations described here, although ovine isolates will be more fastidious to grow in the laboratory [19].

1. Middlebrook 7H9 medium: Dissolve 4.7 g of Difco™ 271310 Middlebrook 7H9 powder (Becton, Dickinson and Company) in 900 ml distilled H_2O and 2 ml of glycerol. For *Map*, use HCl to adjust the pH to 5.9. If preparing culture media for petri dish applications, microbiological agar (15 g/l) is added. Autoclave at 121 °C for 20 min with slow exhaust. Cool to 50 °C (*see* **Note 1**) in order to add supplements (e.g., Tween™ 80, cycloheximide, albumin–dextrose–catalase (ADC; BBL™ Middlebrook 212352; Becton, Dickinson and Company), oleic acid–albumin–dextrose–catalase (OADC; BBL™ Middlebrook 212240; Becton, Dickinson and Company), mycobactin J (Allied Monitor, Inc.)) and any desired antibiotic(s) for

selection. Under sterile conditions, add 2.5 ml of 20% Tween™ 80, 10 ml of 1% cycloheximide and 100 ml of ADC (*M. smegmatis*). For *Map*, add 2.5 ml of 20% Tween™ 80, 10 ml of 1% cycloheximide, 1 mg of mycobactin J, and 100 ml of OADC instead of ADC. Tween™ 80 is not added if preparing agar (*see* **Note 2**).

2. Middlebrook 7H10 agar medium: Dissolve 19 g of Difco™ 262710 Middlebrook 7H10 powder (Becton, Dickinson and Company) in 900 ml distilled H_2O and 5 ml of glycerol. Autoclave at 121 °C for 20 min with slow exhaust. Cool to 50 °C and add supplements and/or antibiotic(s) as listed above (*see* Subheading 2, **item 1**).
3. Mycobacterial phage (MP) buffer: For *M. smegmatis*, the standard composition is 50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 10 mM $MgSO_4 \cdot 7H_2O$; and 2 mM $CaCl_2$. For *Map*, the components are 50 mM Tris–HCl, pH 7.6; 150 mM NaCl; 10 mM $MgCl_2$; and 2 mM $CaCl_2$. These both can be stored up to 1 year at room temperature.
4. Phage soft agar: media is prepared as stated above (*see* Subheading 2, **item 1** or **2**) depending on the bacterial source except that 7 g/l of microbiological agar is added.
5. Adsorption-stop buffer: MP buffer containing 20 mM sodium citrate and 0.2% Tween™ 80. This solution is added to prevent further phage infections after completion of the adsorption time.
6. 50% glycerol-PBS freezing solution: 2 mM NaH_2PO_4, 4 mM Na_2HPO_4, 4 mM NaCl, and 50% glycerol (vol/vol).

3 Methods

3.1 Mycobacterial Culture

1. Inoculate a 2-l flask containing 500 ml of Middlebrook 7H9 at pH 5.9 supplemented with 0.2% glycerol, OADC, 0.05% Tween™ 80, 1 mg mycobactin J and 0.01% cycloheximide (*see* Subheading 2, **item 1**). Use a single, well isolated colony of the *Map* strain of choice for the inoculum.
2. Incubate culture stagnant for 4 weeks checking optical density at 540 nanometers ($O.D._{540nm}$) after the third and fourth week.
3. Harvest 50-ml aliquots of cultures in mid-log phase ($O.D._{540\ nm} = 0.4$ to 0.75) by centrifugation at 4420 × *g* for 25 min. This optical density equates to approximately 2.0×10^8 cfu/ml.
4. If preparing a fresh stock of the phagemid, start the phagemid preparation protocol (Subheading 3.2) during the fourth week of growing the *Map* culture.

3.2 Phagemid Preparation

The phagemid ϕMycoMarT7 must be propagated and titered using freshly grown *M. smegmatis* cells at 30 °C.

1. Grow approximately 10 ml of *M. smegmatis* mc^2155 shaking overnight at 37 °C in Middlebrook 7H9 media supplemented with 0.2% glycerol, ADC, 0.05% Tween™ 80 and 0.01% cycloheximide (*see* Subheading 2, **item 1**).
2. From a known ϕMycoMarT7 titer number, make appropriate dilutions in MP buffer. For example, phagemid at a titer of 10^{10} plaque-forming units (PFU)/ml will usually have a "lace" pattern (plaques are touching) at 10^{-6} that is used for propagation and countable plaques are determined at the 10^{-8} dilution. The final dilution should be made several times depending on how much phage needs to be propagated (*see* **Note 3**).
3. Mix the *M. smegmatis* mc^2155 culture with the phagemid dilution in a 2:1 volume ratio (e.g., 2 ml of culture with 1 ml diluted phagemid).
4. Incubate standing at 30 °C for 30 min to allow for adsorption.
5. Add 300 μl of the phagemid–cell mixture to 3.5 ml of phage soft agar (*see* Subheading 2, **item 4**) and gently mix by rolling the tube with your palms. It is important to keep the phage soft agar at 56 °C until ready to use in a glass tube in a heat block.
6. Pour each tube of phage soft agar containing phagemid–cell mixture onto separate Middlebrook 7H9 agar plates (*see* **Note 4**). Quickly rotate the plate in a circular motion to evenly spread the agar over the entire surface. Once solidified, invert the plates to prevent condensation.
7. Incubate at 30 °C overnight or until plaques are visible (2–3 days).
8. Once plaques are produced and clearly visible, flood each plate with 5 ml of MP buffer. These plates are then incubated at 4 °C for 2–4 h followed by incubation at 37 °C for 1–2 h (*see* **Note 5**).
9. Collect the liquid from each plate by pipetting or scraping the top agar off with an L-spreader and pooling in an Oak Ridge centrifuge tube. It is important to use as little buffer as possible so as to not dilute the new stock.
10. Centrifuge for 30 min at 23,300 × *g* at 4 °C to remove agar pieces and bacterial debris. The phagemid stock should now be clarified.
11. Filter phage stock with a 0.45 μm filter and store at 4 °C (crude phage stock). To further purify the crude phage stock, if needed, ultracentrifuge at 82,705 × *g* for 6 h at 4 °C using a SW28 rotor. Dissolve pellet in MP buffer 200 μl and wash with 50–100 μl of 2× MP buffer (*see* **Note 6**).

12. Titer new phage stock to determine its concentration (*see* **Note 7**). This is done by plating phagemid dilutions on *M. smegmatis* cells. Ideally, the titer should be at least 1×10^{10} PFU per ml or better.

3.3 Transductions

A schematic representation of the entire transduction and collection procedure encompassing Subheadings 3.3–3.5 are depicted in Fig. 1.

1. Add 1 ml of MP buffer to the *Map* bacteria concentrated from **step 3** of Subheading 3.1.
2. Infect *Map* (1.0×10^7 CFU in 0.1 ml) with 0.1 ml of φMycoMarT7 at 1.0×10^8 PFU (*see* **Note 8**) to have a multiplicity of 10 PFU per bacterium, based on the phage titer determined at the permissive temperature for propagation (30 °C). Set up multiple aliquots if plating multiple plates (1 for each plate). Also have separate control tubes with just bacteria and phage.
3. Leave the *Map* and phagemid mixture incubating at 37 °C overnight. At the nonpermissive temperature (37 °C), the phage acts as a nonlytic transducing phage for transposon delivery into *Map*.

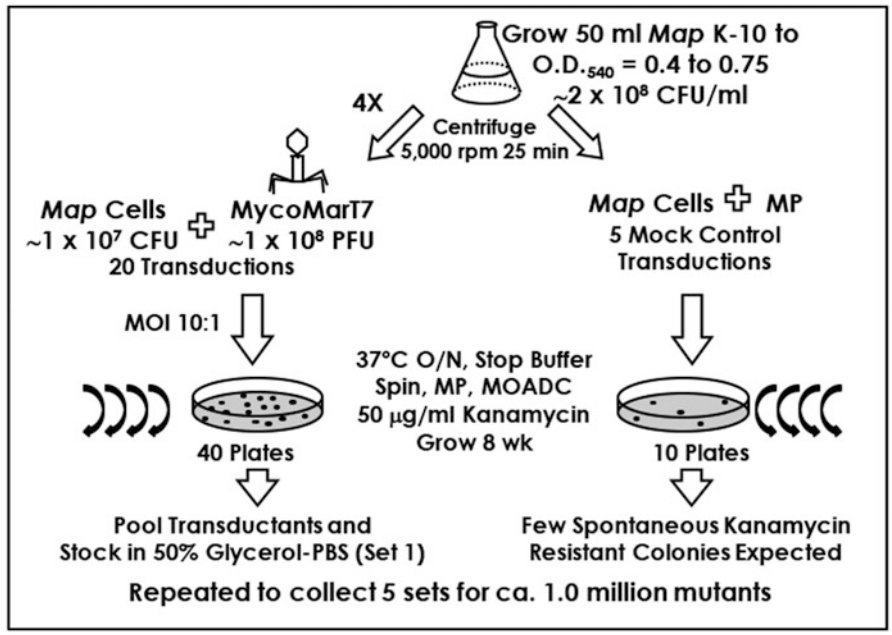

Fig. 1 Flow diagram showing the steps on how to generate a Map MycoMarT7 mutant library. The left side of the flow diagram shows the transduction sets to be pooled and stocked while the right side shows the mock transductions (without phage) to determine percentage of spontaneous kanamycin resistance

4. Add an equal volume of stop buffer, wait 5 min and immediately proceed to plating in the next section.

3.4 Plating Transposon Mutants

1. Independent transductions can be performed and plated separately to obtain a collection of insertional mutants. Alternatively, colonies can be pooled from several independent transductions to obtain a highly representative insertional library.
2. Plate 0.1 ml volumes of the stopped transduction mixture on Middlebrook 7H10 plates supplemented with 0.5% glycerol, 0.01% cycloheximide, 1 mg of mycobactin J and OADC without Tween 80, but containing either 50 μg/ml kanamycin. Use a bent glass rod "hockey stick" or L-spreader to disperse the aliquot over the entire surface of the plate (*see* **Note 4**).
3. Individual kanamycin-resistant transductants are randomly picked after an 8 week incubation period at 37 °C.
4. The entire library can be harvested and stocked in 50% glycerol-PBS freezing solution. The library can be stored in 0.5-ml aliquots at −80 °C.

3.5 Harvesting Colonies Representing Transposon Mutants

1. Colonies can be picked individually or harvested together depending on the goal of the experiment.
2. If picking and stocking individual colonies separately, these can be picked using a sterile needle into 96-well plates containing Middlebrook 7H9 media (0.2 ml).
3. If harvesting together, flood the plates with either MP buffer or Middlebrook 7H9 media and concentrate mutants by centrifugation.
4. DNA can then be extracted for Tn-seq analysis or individually stocked mutants can be screened for various phenotypic traits.

4 Notes

1. The ADC/OADC supplement is purchased commercially, filtered through a 0.2 μm filter assembly and added to the autoclaved-sterilized Middlebrook 7H9 media. Note that ADC/OADC is only added after the media has cooled below 50 °C. Bacteriological media for plating transductions should be prepared fresh 2 days before the experiment and allowed to dry in a 37 °C incubator to prevent water accumulation from condensation. Alternatively for Middlebrook 7H9 and 7H10 media preparation, ADC/OADC can be made from scratch with additional details found in Chacon et al. [20] and Larsen et al. [21]. For the growth of *M. smegmatis* mc^2155 and *Map* K-10, the addition of catalase and cycloheximide to these media is optional.

2. Tween™ 80 helps prevent clumping, which is not necessary when plating on solid media.
3. High titer lysates are used for propagation of phage when large amounts of phage are needed for transductions. Examples include replenishing working stocks, phage purification, or phage DNA isolation.
4. Solid media petri plates come in various sizes but the most common is 100 mm × 15 mm (60 cm^2 area) can be sealed with paraffin to prevent drying.
5. The phagemid will diffuse into the MP buffer during the overnight incubation. The plates can be kept stagnant or on a slow rocking platform. The phage soft agar will shrink at 4 °C and then expand under the 37 °C temperature to help release the phage Larsen et al. [21].
6. Keep the mycobacteriophage in two stock vials: working stock and back-up stock. Then only use from the working stock bottles and keep the back-up vial only for repropagation of phage when the working stock is depleted or has developed a problem such as contamination.
7. All phage stocks must be kept in the refrigerator at 4 °C. If the phage must be taken out of the refrigerator for any length of time, then the phage should be placed on ice. Temperature variations will cause a phage titer to decrease.
8. When using the phage stocks, extreme care must be used to prevent contamination of the stocks. We suggest wearing alcohol-rinsed latex gloves, wiping the phage stock bottle off with alcohol tissue and allowing it to dry thoroughly, using fresh sterile pipet tips every time an aliquot is taken from the phage stock bottle, and wrapping the bottle lid with paraffin before returning to 4 °C storage.

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References

1. Foley-Thomas EM, Whipple DL, Bermudez LE, Barletta RG (1995) Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. Microbiology 141(Pt 5):1173–1181
2. Harris NB, Feng Z, Liu X, Cirillo SL, Cirillo JD, Barletta RG (1999) Development of a transposon mutagenesis system for *Mycobacterium avium subsp. paratuberculosis*. FEMS Microbiol Lett 175(1):21–26. doi:S0378-1097(99)00170-6 [pii]
3. Cirillo JD, Barletta RG, Bloom BR, Jacobs WR Jr (1991) A novel transposon trap for

mycobacteria: isolation and characterization of IS1096. J Bacteriol 173(24):7772–7780

4. Rathnaiah G, Lamont EA, Harris NB, Fenton RJ, Zinniel DK, Liu X, Sotos J, Feng Z, Livneh-Kol A, Shpigel NY, Czuprynski CJ, Sreevatsan S, Barletta RG (2014) Generation and screening of a comprehensive *Mycobacterium avium* subsp. *paratuberculosis* transposon mutant bank. Front Cell Infect Microbiol 4:144. https://doi.org/10.3389/fcimb.2014.00144
5. Ghosh P, Wu CW, Talaat AM (2013) Key role for the alternative sigma factor, SigH, in the intracellular life of *Mycobacterium avium subsp. paratuberculosis* during macrophage stress. Infect Immun 81(6):2242–2257. https://doi.org/10.1128/IAI.01273-12
6. Shippy DC, Lemke JJ, Berry A, Nelson K, Hines ME 2nd, Talaat AM (2017) Superior protection from live-attenuated vaccines directed against Johne's disease. Clin Vaccine Immunol 24(1). https://doi.org/10.1128/CVI.00478-16
7. Chen JW, Scaria J, Chang YF (2012) Phenotypic and transcriptomic response of auxotrophic *Mycobacterium avium subsp. paratuberculosis leuD* mutant under environmental stress. PLoS One 7(6):e37884. https://doi.org/10.1371/journal.pone.0037884
8. Park KT, Dahl JL, Bannantine JP, Barletta RG, Ahn J, Allen AJ, Hamilton MJ, Davis WC (2008) Demonstration of allelic exchange in the slow-growing bacterium *Mycobacterium avium* subsp. *paratuberculosis*, and generation of mutants with deletions at the *pknG*, *relA*, and *lsr2* loci. Appl Environ Microbiol 74(6):1687–1695. https://doi.org/10.1128/AEM.01208-07. AEM.01208-07 [pii]
9. Park KT, Allen AJ, Barrington GM, Davis WC (2014) Deletion of *relA* abrogates the capacity of *Mycobacterium avium paratuberculosis* to establish an infection in calves. Front Cell Infect Microbiol 4:64. https://doi.org/10.3389/fcimb.2014.00064
10. Faisal SM, Chen JW, Yan F, Chen TT, Useh NM, Yan W, Guo S, Wang SJ, Glaser AL, McDonough SP, Singh B, Davis WC, Akey BL, Chang YF (2013) Evaluation of a *Mycobacterium avium* subsp. *paratuberculosis leuD* mutant as a vaccine candidate against challenge in a caprine model. Clin Vaccine Immunol 20(4):572–581. https://doi.org/10.1128/CVI.00653-12
11. Rathnaiah G, Bannantine JP, Bayles DO, Zinniel DK, Stabel JR, Grohn YT, Barletta RG (2016) Analysis of *Mycobacterium avium subsp. paratuberculosis* mutant libraries reveals loci-dependent transposition biases and strategies to novel mutant discovery. Microbiology. https://doi.org/10.1099/mic.0.000258
12. Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE (2007) Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A 104(26):11038–11043. https://doi.org/10.1073/pnas.0610746104. 0610746104 [pii]
13. Wu CW, Livesey M, Schmoller SK, Manning EJ, Steinberg H, Davis WC, Hamilton MJ, Talaat AM (2007) Invasion and persistence of *Mycobacterium avium* subsp. *paratuberculosis* during early stages of Johne's disease in calves. Infect Immun 75(5):2110–2119. https://doi.org/10.1128/IAI.01739-06. IAI.01739-06 [pii]
14. Bannantine JP, Everman JL, Rose SJ, Babrak L, Katani R, Barletta RG, Talaat AM, Grohn YT, Chang YF, Kapur V, Bermudez LE (2014) Evaluation of eight live attenuated vaccine candidates for protection against challenge with virulent *Mycobacterium avium* subspecies *paratuberculosis* in mice. Front Cell Infect Microbiol 4:88. https://doi.org/10.3389/fcimb.2014.00088
15. Shin SJ, Wu CW, Steinberg H, Talaat AM (2006) Identification of novel virulence determinants in *Mycobacterium paratuberculosis* by screening a library of insertional mutants. Infect Immun 74(7):3825–3833. https://doi.org/10.1128/IAI.01742-05. 74/7/3825 [pii]
16. Bardarov S, Kriakov J, Carriere C, Yu S, Vaamonde C, McAdam RA, Bloom BR, Hatfull GF, Jacobs WR Jr (1997) Conditionally replicating mycobacteriophages: a system for transposon delivery to *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 94(20):10961–10966
17. Sassetti CM, Boyd DH, Rubin EJ (2001) Comprehensive identification of conditionally essential genes in mycobacteria. Proc Natl Acad Sci U S A 98(22):12712–12717. https://doi.org/10.1073/pnas.231275498
18. Rubin EJ, Akerley BJ, Novik VN, Lampe DJ, Husson RN, Mekalanos JJ (1999) In vivo transposition of mariner-based elements in enteric bacteria and mycobacteria. Proc Natl Acad Sci U S A 96(4):1645–1650
19. Reddacliff LA, Nicholls PJ, Vadali A, Whittington RJ (2003) Use of growth indices from radiometric culture for quantification of sheep strains of *Mycobacterium avium* subsp. *paratuberculosis*. Appl Environ Microbiol 69(6):3510–3516

20. Chacon O, Bermudez LE, Zinniel DK, Chahal HK, Fenton RJ, Feng Z, Hanford K, Adams LG, Barletta RG (2009) Impairment of D-alanine biosynthesis in *Mycobacterium smegmatis* determines decreased intracellular survival in human macrophages. Microbiology 155(Pt 5):1440–1450. https://doi.org/10.1099/mic.0.024901-0

21. Larsen MH, Biermann K, Jacobs WR Jr (2007) Laboratory maintenance of *Mycobacterium tuberculosis*. Curr Protoc Microbiol. Chapter 10:Unit 10A 11. https://doi.org/10.1002/9780471729259.mc10a01s6